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Two S-Iamide Peptides, AKSGFVRIamide and VSSFVRIamide, Isolated from an Annelid, *Perinereis vancaurica*

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Abstract. Two peptides, H-Ala-Lys-Ser-Gly-Phe-Val-Arg-Ile-NH_2 (AKSGFVRIamide), and H-Val-Ser-Ser-Phe-Val-Arg-Ile-NH_2 (VSSFVRIamide) were isolated from a polychaete annelid, *Perinereis vancaurica*. Both the peptides evoked rhythmic contractions in the esophagus of *Perinereis* with a threshold as low as 10^{-10}–10^{-9} *M*, suggesting that the peptides may be involved in the regulation of gut motility of the animal. The sequences of these peptides are very similar to those of other S-Iamide family peptides which have been previously isolated from an echiuroid worm and some molluscs. In particular, the sequence of VSSFVRIamide is identical to that of an echiuroid S-Iamide peptide. All of the molluscan and echiuroid S-Iamide peptides, as well as the annelid peptides, were found to produce contractions in the esophagus of *Perinereis*. On the other hand, the annelid S-Iamide peptides, as well as the molluscan and echiuroid peptides, were found to inhibit or potentiate contractions elicited by electrical stimulation in echiuroid and molluscan muscles. S-Iamide peptides may be a typical neuropeptide family distributed interphyletically in the Protostomia.

Introduction

In annelids, pharmacological studies have been extensively done on the actions of classical transmitters such as 5-hydroxytryptamine, epinephrine, norepinephrine and dopamine mainly on somatic muscles, and these substances have been suggested to be present in the central and peripheral nervous systems (for review, Tashiro and Kuriyama, 1978). In addition, bioactive peptides found in vertebrates and other phyla of invertebrates have been suggested to be present in annelids (Carraway *et al.*, 1982; Engelhardt *et al.*, 1982; Dhainaut-Courtois *et al.*, 1985; Diaz-Miranda *et al.*, 1991, 1992).

Many peptides are known in vertebrates, especially in mammals which control the motility of the gut (Holmgren, 1989). However, few gut motility-controlling peptides have been reported for invertebrates. Immunohistochemical or immunochemical studies have suggested that some vertebrate neuropeptides, such as enkephalin, β-endorphin (Alumets *et al.*, 1979), substance P (Dhainaut-Courtois *et al.*, 1985; Kaloustian and Edmands, 1986), cholecystokinin/gastrin, β-MSH (Engelhardt *et al.*, 1982; Dhainaut-Courtois *et al.*, 1985) and neurotensin (Carraway *et al.*, 1982) may be present in annelids. Kaloustian and Edmands (1986) reported that substance P stimulated the rate of spontaneous contraction of intestinal tissues of the earthworm *Lumbricus terrestris*. It has also been shown that a tetrapeptide (WMDFamide) related to cholecystokinin/gastrin has excitatory effects on the anterior intestine of a polychaete, *Chaetopterus variopedatus* (Anctil *et al.*, 1984). Apart from immunocytochemical and pharmacological studies, most investigations of bioactive peptides in annelids have centered on those involved in reproductive events (Thorndyke, 1989). Thus, reports on authentic bioactive peptides involved in the regulation of gut-motility of annelids are very few in number.

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Recently, Krajniak and Price (1990) showed the presence of FMRFamide which was first identified in a mollusc as a cardioexcitatory neuropeptide in the polychaete *Nereis virens* (Price and Greenberg, 1977). Krajniak and Greenberg (1992) showed that immunoreactive FMRFamide was present in various tissues including the gut in *Nereis*, and that FMRFamide had a relaxing action on both the spontaneously active and electrically stimulated esophagus, suggesting the involvement of the tetrapeptide in the control of gut-motility. Furthermore, FMRFamide and its related peptides have been shown to be present in other annelid species such as *Nereis diversicolor* (Baratte *et al.*, 1991) and *Hirudo medicinalis* (Evans *et al.*, 1991).

In the present study, we isolated and sequenced two bioactive peptides, AKSGFVRIamide and VSSFVRIamide, from the polychaete *Perinereis vancaurica*, which induced contraction of the isolated esophagus of the animal. These peptides were found to be members of the S-Iamide peptide family (Ikeda *et al.*, 1991; Muneoka and Kobayashi, 1992). The name S-Iamide peptide was given after the common structure, -SSFVRIamide. Kuroki *et al.* (1992) first isolated one of the S-Iamide peptides, LSSFVRIamide, from the prosobranch mollusc *Fusinus ferrugineus*, and up to the present, S-Iamide peptides have been found not only in molluscs but also in an echiuroid worm (Ikeda *et al.*, 1991). We also examined the effects of several S-Iamide peptides on some invertebrate muscle tissues including the esophagus of *Perinereis*.

Materials and Methods

Purification

Perinereis vancaurica tetradentata are commercially available as fishing bait. Approximately 380 worms (500 g) were rinsed twice with artificial seawater (ASW), blotted lightly with tissue paper and boiled for 10 min in 4 volumes of 4% acetic acid (21). The animals were homogenized in 4% acetic acid by using a Waring blender and a Polytron. The homogenate was centrifuged (15,000 × *g*, 40 min, 4°C), and the resulting precipitate was again homogenized and centrifuged. The two supernatants were pooled and concentrated to a volume of about 100 ml by using a rotary evaporator (40°C). To the concentrated supernatant, 1/10 volume of 1 *N* HCl was added, and the precipitated material was centrifuged off (15,000 × *g*, 40 min, 4°C). Next, the supernatant was forced through two disposable C-18 cartridges in series (Mega Bond-Elut, Varian). The retained material was eluted with 50% methanol. The eluate was concentrated, loaded on a C-18 reversed phase HPLC column (CAPCELL-PAK, Shiseido; 10 mm × 250 mm), and eluted with a linear gradient of 0–60% acetonitrile (ACN) in 0.1% trifluoroacetic acid (TFA) for 120 min at a flow rate of 1 ml/min. The chromatography was monitored at 220 nm. Aliquots of 2 ml-fractions were evaporated to dryness, and the residues were dissolved in ASW and bioassayed on an isolated esophagus of *Perinereis* as described below. Two contractile peaks were detected. The fractions of each active peak were concentrated and subjected to HPLC using another C-18 reversed phase column (ODS-80TM, Tosoh; 4.6 mm × 150 mm) with a linear gradient of 10–20% ACN for one activity and 15–25% ACN for the other in 0.1% TFA (0.5 ml/min). Active fractions obtained from each HPLC were then loaded onto a cation-exchange column (SP-5PW, Tosoh; 7.5 mm × 75 mm) and eluted in a linear gradient of 0–0.7 *M* NaCl in 10 m*M* phosphate buffer (pH 7.1) for 70 min at a flow rate of 0.5 ml/min. Then, the active substances identified as single peaks on the cation-exchange HPLC were chromatographed on the ODS-80TM column with a linear gradient of 10–16% ACN and 15–25% ACN, respectively, and finally purified on the same column with an isocratic elution of 17% and 19% ACN.

The two purified substances were subjected to amino acid sequence analysis by automated Edman degradation with a gas-phase sequencer (Shimazu PSQ-1). The results of the chemical analyses suggested that the substances were members of the S-Iamide peptide family. Therefore, the two peptides having the suggested structures were synthesized by a manual method followed by an HF-anisol cleavage and purified by reversed-phase HPLC. Then, the synthetic peptides were compared with native ones in the behavior on HPLC and in the bioactivity on the *Perinereis* esophagus.

Bioassay

The contractile activities of the native and synthetic substances were examined on the isolated esophagus of *Perinereis*. The method for contraction recording was essentially the same as that reported by Krajniak and Greenberg (1992). Both ends of the isolated esophagus were ligated with two cotton threads, one being secured to a stationary rod at the bottom of a trough (2 ml) and the other connected to a force-displacement transducer (NEC San-ei Instruments).

The saline in the trough was constantly aerated through a syringe needle connected with an air pump to ensure uniform distribution of applied substances (dissolved in 0.1 ml saline) in the trough. In the present study, we did not apply electrical stimulation but just monitored inductivity of spontaneous contractions of the esophagus by test substances. For examination of the bioactivity of the material retained by the C-18 cartridges, two more assay systems, the inner circular body-wall muscle of an echiuroid worm *Urechis unicinctus* (Ikeda *et al.*, 1991), and the radula retractor muscle of a prosobranch mollusc

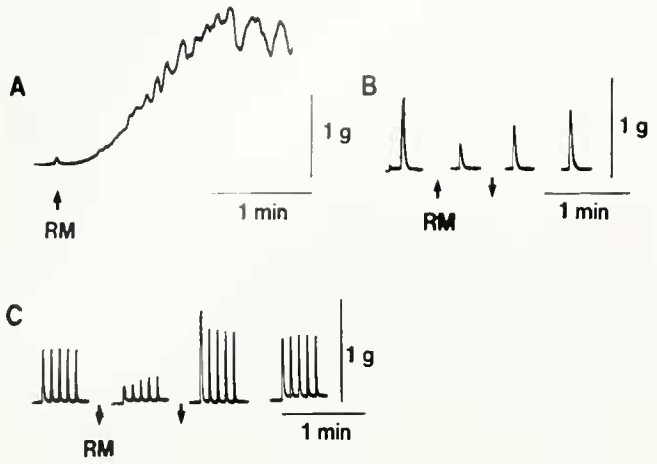

Figure 1. Effects of the retained materials (RM) on the three muscle systems. (A) the esophagus of *Perinereis*. (B) twitch contractions of the inner circular body-wall muscle of *Urechis*. The twitch contraction was produced by an electrical pulse (20 V, 3 ms). (C) twitch contractions of the radula retractor of *Rapana* The twitch contractions were produced by a train of electrical pulses (15 V, 1 ms, 0.2 Hz, 5 pulses). In each case, 1/1000 of total RM, which corresponded with extracts from 0.4 worm, was applied to the assay system. The upward arrows indicate application of RM to the tissue. The downward arrows indicate washing-out of the RM.

Rapana thomasiana (Muneoka *et al.*, 1991), were employed. In these cases, electrical pulses of stimulation were applied to the preparations.

Pharmacology

The methods used in the pharmacological experiments were basically the same as those in the bioassay experiments. In these experiments, we used three kinds of muscles, the esophagus of *Perinereis*, the anterior byssus retractor muscle (ABRM) of the bivalve mollusc *Mytilus edulis* and the radula retractor muscle of the prosobranch mollusc *Fusinus ferrugineus*.

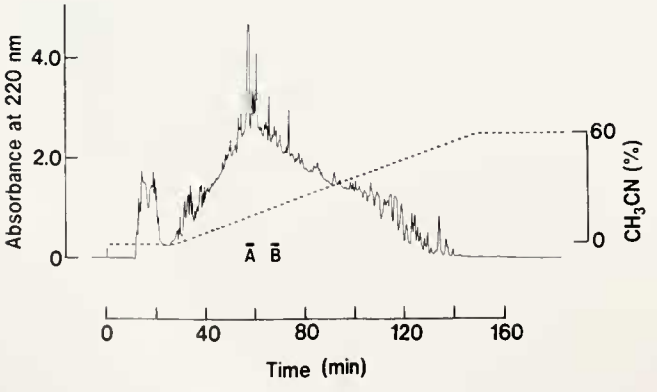

Figure 2. HPLC profile of the retained materials (RM) on a reversed phase column. The RM loaded onto the column was eluted with a linear gradient of ACN concentration (0–60%/120 min) in 0.1% TFA (pH 2.2) at a flow rate of 1 ml/min and collected in 60 fractions of 2 ml each. Aliquots (10 μl = 1/200) of each fraction were evaporated to dryness, dissolved in ASW and applied to the *Perinereis* esophagus. The contractile peaks were indicated by the horizontal bars (A and B).

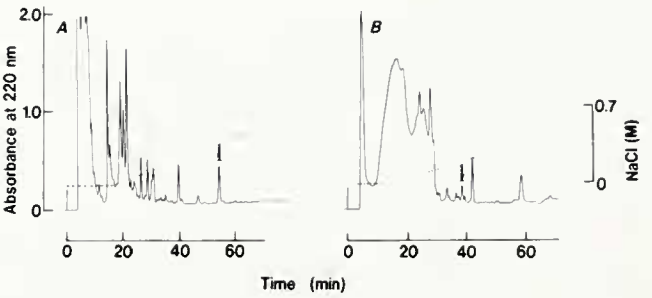

Figure 3. HPLC profiles of active fractions (A and B in Fig. 2) on a cation-exchange column. Elution was performed in a 70-min linear gradient of 0–0.7 *M* NaCl in 10 m*M* phosphate buffer (pH 7.1) at a flow rate of 0.5 ml/min (collected in 1-ml fractions). The activities (A and B) were detected in respective peaks indicated by arrows.

Salines

The saline used for *Perinereis* and *Urechis* muscles was ASW of the following composition: 445 m*M* NaCl, 55 m*M* $MgCl_2$, 10 m*M* $CaCl_2$, 10 m*M* KCl, 10 m*M* Tris-HCl; pH 7.6. For the *Rapana* and *Fusinus* muscles, low Mg-ASW (20 m*M* $MgCl_2$) was used. The low-Mg ASW was prepared by replacing a part of $MgCl_2$ in the normal ASW with osmotically equivalent NaCl.

Results

The retained material (RM) eluted with 50% methanol was examined for its biological action on three muscle systems, the esophagus of *Perinereis*, the inner circular body-wall muscle of *Urechis* and the radula retractor muscle of *Rapana* (Fig. 1). The RM elevated a basal tone with rhythmic small contractions in the esophagus of *Perinereis* and exerted inhibitory effects on twitch contractions evoked by electrical stimulations in the latter two muscles. After the test solution was replaced with normal ASW, the contractions of *Rapana* radula retractor became greater than the control contractions and then returned to the control level. We decided to purify at first the substance which elicited contractions of the *Perinereis* esophagus.

At the first step of HPLC, two contractile peaks (peak A and B) were found. They were eluted at approximately 15% and 20% ACN, respectively (Fig. 2). At the second step, active substances of peak A and B were eluted at 13% ACN and 19% ACN, respectively (data not shown). Then, the fractions containing active substance A and B were respectively subjected to the cation-exchange HPLC

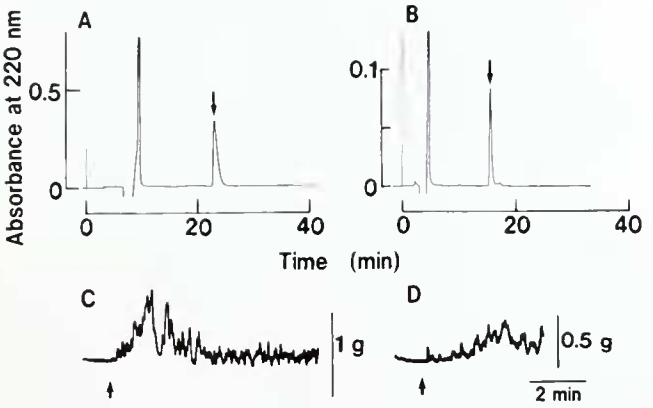

Figure 4. Final purification by HPLC using a reversed-phase column (A, B) and the action of each purified substance on the *Perinereis* esophagus (C, D). Isocratic elution with 17% ACN (A) and 19% ACN (B) in 0.1% TFA at a flow rate of 0.3 ml/min. Aliquots (1/100) of the purified substances were dissolved in ASW and applied to the isolated esophagus at the time indicated by arrows (C, D).

(Fig. 3). The active substances appeared to be eluted as single peaks around 0.35 *M* NaCl (A) and 0.25 *M* NaCl (B). The final purification was performed on the C-18 column with an isocratic elution of 17% ACN (A) and 19% ACN (B) (Fig. 4). The respective single peaks with OD at 220 nm of 0.303 (A) and 0.085 (B) were eluted at 23 min and 16 min after injection. The purified substances (1/100) elicited rhythmic contractions of the esophagus (Fig. 4C, D).

Amino acid sequence analysis of the purified substances (A and B) revealed the structure to be the octapeptide Ala (216)-Lys (43.3)-Ser (8.7)-Gly (7.0)-Phe (5.9)-Val (1.7)-Arg (1.3)-Ile (+) and the heptapeptide Val (181.5)-Ser (66.0)-Ser (45.8)-Phe (118.6)-Val (150.2)-Arg (28.2)-Ile (0.9), respectively (the figures are expressed in pmoles). The peptides of the respective sequences with C-terminus amidated were synthesized, and HPLC profiles of the synthetic peptides were compared with those of native ones. The synthetic and native peptides showed identical retention times on the C-18 reversed-phase column and the cation-exchange column (data not shown). Furthermore, the mixture of the synthetic and native peptides was eluted as a single peak on each column (Fig. 5). The respective synthetic peptides evoked contraction of the *Perinereis* esophagus in the similar manner to the corresponding native peptides (Figs. 6, 7). The threshold concentrations for the synthetic peptides to evoke contraction were found to be between 10^{-10} *M* and 10^{-9} *M* for both peptides.

Thus, the structures of substance A and B were concluded to be AKSGFVRIamide and VSSFVRIamide, respectively. Both AKSGFVRIamide and VSSFVRIamide were members of S-Iamide peptides which had been purified from one echiuroid and four mollusks (Table 1).

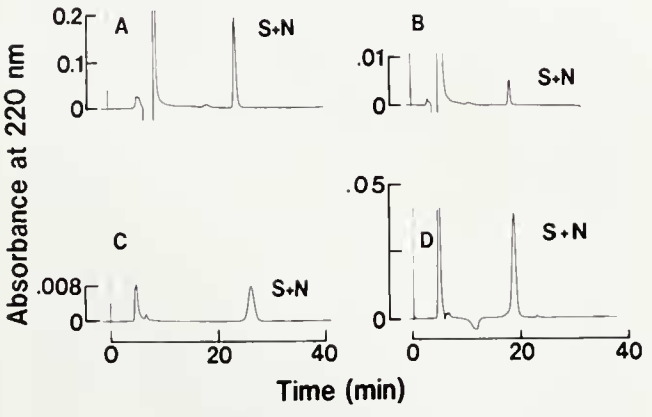

Figure 5. HPLC profiles of mixtures of native and synthetic peptides. AKSGFVRIamide (A) and VSSFVRIamide (B) on a reversed phase column with an isocratic elution of 17% ACN and 19% ACN at a flow rate of 0.3 ml/min. AKSGFVRIamide (C) and VSSFVRIamide (D) on a cation-exchange column with an isocratic elution of 0.27 *M* NaCl and 0.14 *M* NaCl in 10 m*M* phosphate buffer (pH 7.1) at a flow rate of 0.5 ml/min.

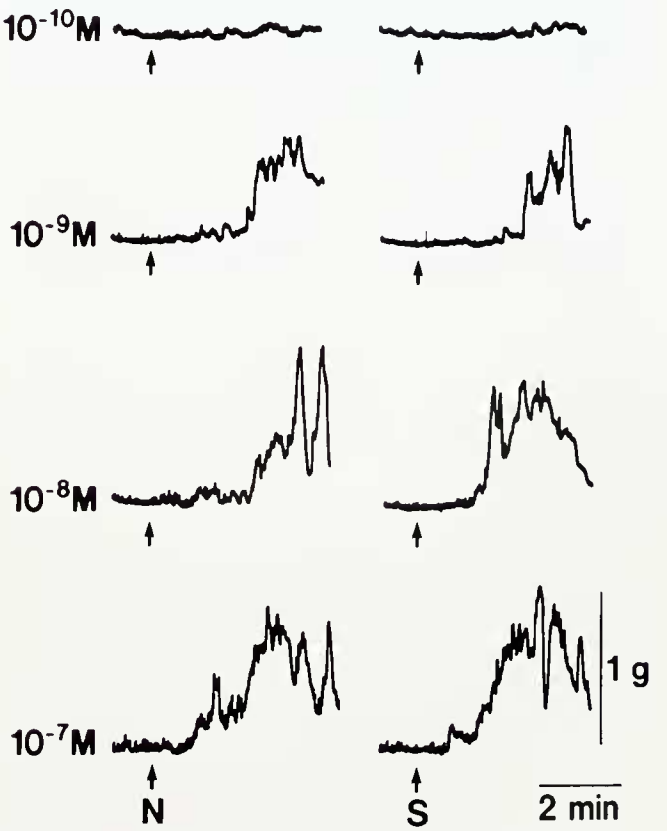

Figure 6. Comparison of the bioactivities of the native (N) and synthetic (S) peptides (AKSGFVRIamide) on the isolated esophagus of *Perinereis*. The peptide solutions were applied at the time indicated by arrows. The concentration of the native peptide was estimated by comparing its peak height on HPLC with that of the synthetic peptide.

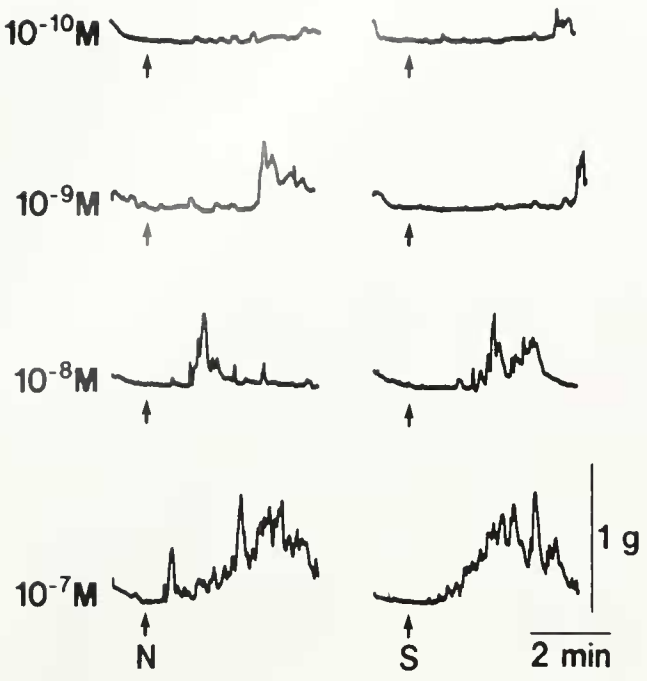

Figure 7. Comparison of the bioactivities of the native (N) and synthetic (S) peptides (VSSFVRIamide) on the isolated esophagus of *Perinereis*. The peptide solutions were applied at the time indicated by arrows. The concentration of the native peptide was estimated by comparing its peak height on HPLC with that of the synthetic peptide.

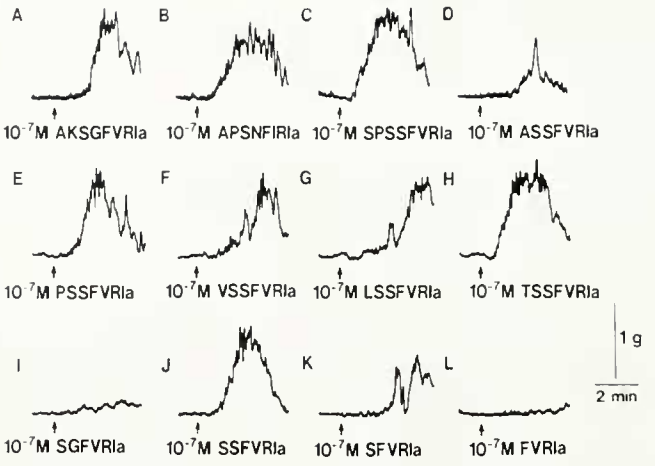

Figure 8. The actions of 10^{-7} *M* of various S-Iamide peptides and some fragment peptides on the isolated *Perinereis* esophagus. Each peptide was applied at the time indicated by arrows.

These S-Iamide peptides and some fragment peptides were examined on the *Perinereis* esophagus (Fig. 8). All of the S-Iamide family peptides and the fragment peptides more or less elicited rhythmic contractions of the esophagus at 10^{-7} *M*.

The biological activities of the two S-Iamide peptides isolated from *Perinereis* were examined on three muscle systems, the inner circular body-wall muscle of *Urechis* (Fig. 9), the ABRM of *Mytilus* (Fig. 10) and the radula retractor muscle of *Fusinus* (Fig. 11). In the *Urechis* muscle, the twitch contraction evoked by electrical stimulation was inhibited by the S-Iamide peptides; AKSGFVRIamide was less potent than VSSFVRIamide (Fig. 9). The phasic contraction of the *Mytilus* ABRM evoked by repetitive electrical stimulation was inhibited by the peptides (Fig. 10). The effects of these two peptides on *Fusinus* muscle were somewhat complicated. AKSGFVRIamide potentiated twitch contractions at the concentration of 10^{-7} *M*, but inhibited at 10^{-5} *M*. VSSFVRIamide, on the other hand, did not show any augmentation of the twitch contraction, but inhibited at concentrations higher than 10^{-6} *M* (Fig. 11).

Table I

S-Iamide peptides

Phyla	Species	Structures
Annelida	*Perinereis vancaurica*	AKSGFVRIamide
		VSSFVRIamide
Echiura	*Urechis unicinctus*	ASSFVRIamide
		PSSFVRIamide
		VSSFVRIamide
Mollusca	*Fusinus ferrugineus*	LSSFVRIamide
	Helix pomatia	TSSFVRIamide
	Achatina fulica	SPSSFVRIamide
		APSNFIRIamide
	Anodonta cygnea	SGFVRIamide

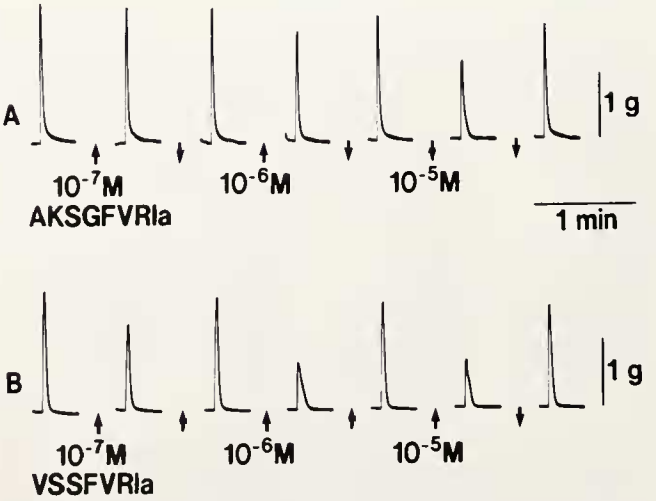

Figure 9. Effects of AKSGFVRIamide and VSSFVRIamide on twitch contraction of the inner circular muscle of the body wall of *Urechis*. The upward arrows indicate application of the peptides. The downward arrows indicate washing-out of the peptides. The twitch contraction was evoked by an electrical pulse (20 V, 3 msec).

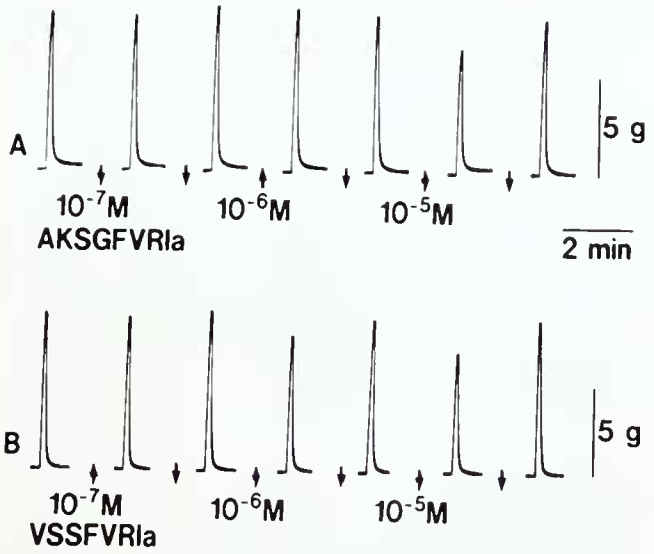

Figure 10. Effects of AKSGFVRIamide and VSSFVRIamide on phasic contraction of the ABRM of *Mytilus*. The upward arrows indicate application of the peptides. The downward arrows indicate washing-out of the peptides. The phasic contraction was evoked by repetitive electrical pulses (15 V, 3 msec, 10 Hz, 50 pulses).

Discussion

The principal aim of this study was to find out authentic bioactive peptides in annelids. We isolated two S-Iamide family peptides, AKSGFVRIamide and VSSFVRIamide, from the polychaete annelid, *Perinereis*. Both the peptides showed a contractile effect on the esophagus of the animal. AKSGFVRIamide is the novel peptide, and VSSFVRIamide has previously been found in the ventral nerve cord of the echiuroid worm, *Urechis*. S-Iamide peptides have been found so far in one species of Echiura and four species of Mollusca (Ikeda *et al.*, 1991; Kuroki *et al.*, 1992; Muneoka and Kobayashi, 1992; in prep. for *Anodonta* S-Iamide peptide), as listed in Table I. Thus, S-Iamide peptides have been proven to be distributed among at least three invertebrate phyla and may range throughout the Protostomia.

The C-termini of the two S-Iamide peptides identified in the current study were concluded to be amidated, though the purified substances were not subjected to fast atom bombardment mass spectrometry. Since the C-termini of all the S-Iamide peptides so far isolated from the echiuroid and molluscs have been known to be amidated, we synthesized AKSGFVRIamide and VSSFVRIamide and compared their behavior on HPLC and contractile activity with those of the purified native peptides. As a result, the identical properties of the native and synthesized peptides were confirmed.

The synthetic tetra- and pentapeptides, FVRIamide and SFVRIamide, showed only a slight activity for induction of the spontaneous contraction in the esophagus of *Perinereis*. However, the synthetic hexapeptide, SSFVRIamide (a common structure for most of the S-Iamide peptides), was active, suggesting that at least six amino acid residues would be important for the expression of the activity of S-Iamide peptides. However, SGFVRIamide which has been isolated from *Anodonta* showed weak contractile activity in the esophagus. The substitution of the amino acid residue, Ser, with Gly seems to be deleterious for contractile activity, and the N-terminal elongation of SGFVRIamide by Ala-Lys might cancel the deleteriousness.

The effect of AKSGFVRIamide on twitch contractions of the radula retractor of *Fusinus* was somewhat complicated. That is, the peptide potentiated the contractions at 10^{-7} *M*, but inhibited at 10^{-5} *M*. The well-known molluscan neuropeptide FMRFamide has been known to potentiate the contractions of the same muscles (Kuroki *et al.*, 1992). Since the C-terminal tetrapeptide sequence of the S-Iamide peptide, -FVRIamide, is closely related to that of FMRFamide, the potentiating effect of 10^{-7} *M* AKSGFVRIamide may be attributable to the FMRFamide-like action of the S-Iamide peptide. The inhibition of the twitch contractions by 10^{-5} *M* of AKSGFVRIamide is probably the original action of the S-Iamide peptide. In this connection, Kuroki *et al.* (1992) reported that another S-Iamide peptide, LSSFVRIamide, isolated from the ganglia of *Fusinus* showed the same dose-dependent actions on the contractions as did AKSGFVRIamide.

The physiological role of AKSGFVRIamide and VSSFVRIamide in *Perinereis* is not elucidated at present. The threshold concentrations of these two S-Iamide peptides to induce contraction of the esophagus were between

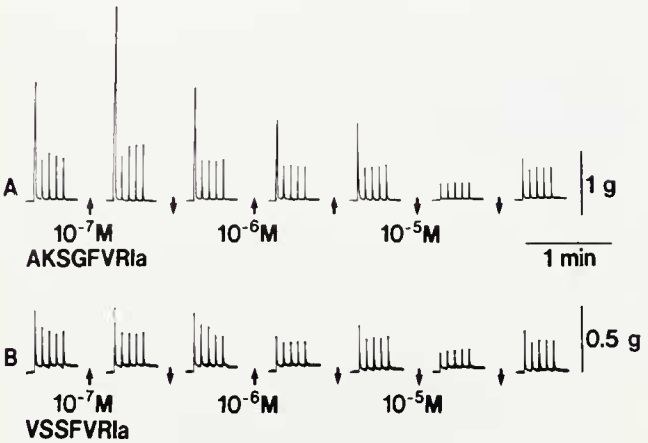

Figure 11. Effects of AKSGFVRIamide and VSSFVRIamide on twitch contractions of the radula retractor muscle of *Fusinus*. The upward arrows indicate application of the peptides. The downward arrows indicate washing-out of the peptides. The twitch contractions were evoked by a train of electrical pulses (15 V, 1 msec, 0.2 Hz, 5 pulses).

10^{-10} *M* and 10^{-9} *M*. It seems to be probable that these S-Iamide peptides are neuropeptides which regulate the gut-motility in the annelid. It has been demonstrated that FMRFamide is present in annelids such as *Nereis virens* (Krajniak and Price, 1990), *Nereis diversicolor* (Baratte *et al.*, 1991) and *Hirudo medicinalis* (Evans *et al.*, 1991) and that the tetrapeptide relaxed spontaneous and electrically-induced contractions of the esophagus (Krajniak and Greenberg, 1992). Thus, the action of FMRFamide on the esophagus of polychaete annelid is opposite to those of the S-Iamide peptides. This was also the case for most of the molluscan muscles examined (Muneoka and Kobayashi, 1992). Therefore, FMRFamide and the S-Iamide peptides may regulate the esophagus-motility in an antagonistic manner, and this regulatory relation may be also applied to the molluscan muscles.

The classical neurotransmitters such as norepinephrine, epinephrine, acetylcholine, 5-hydroxytryptamine and γ-aminobutyric acid also seem to regulate gut-motility in the polychaete annelid, *Chaetopterus variopedatus* (Anctil *et al.*, 1984), and the presence of catecholamines (dopamine, norepinephrine and epinephrine) has been reported in the nervous and intestinal tissues of the same species (Anctil *et al.*, 1990). Thus, peptides such as FMRFamide and the S-Iamide peptides, and classical transmitters seem to regulate the gut-motility harmoniously in annelids. Further study is necessary to reveal the relationship between the physiological role of classical transmitters and peptides.

Acknowledgments

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